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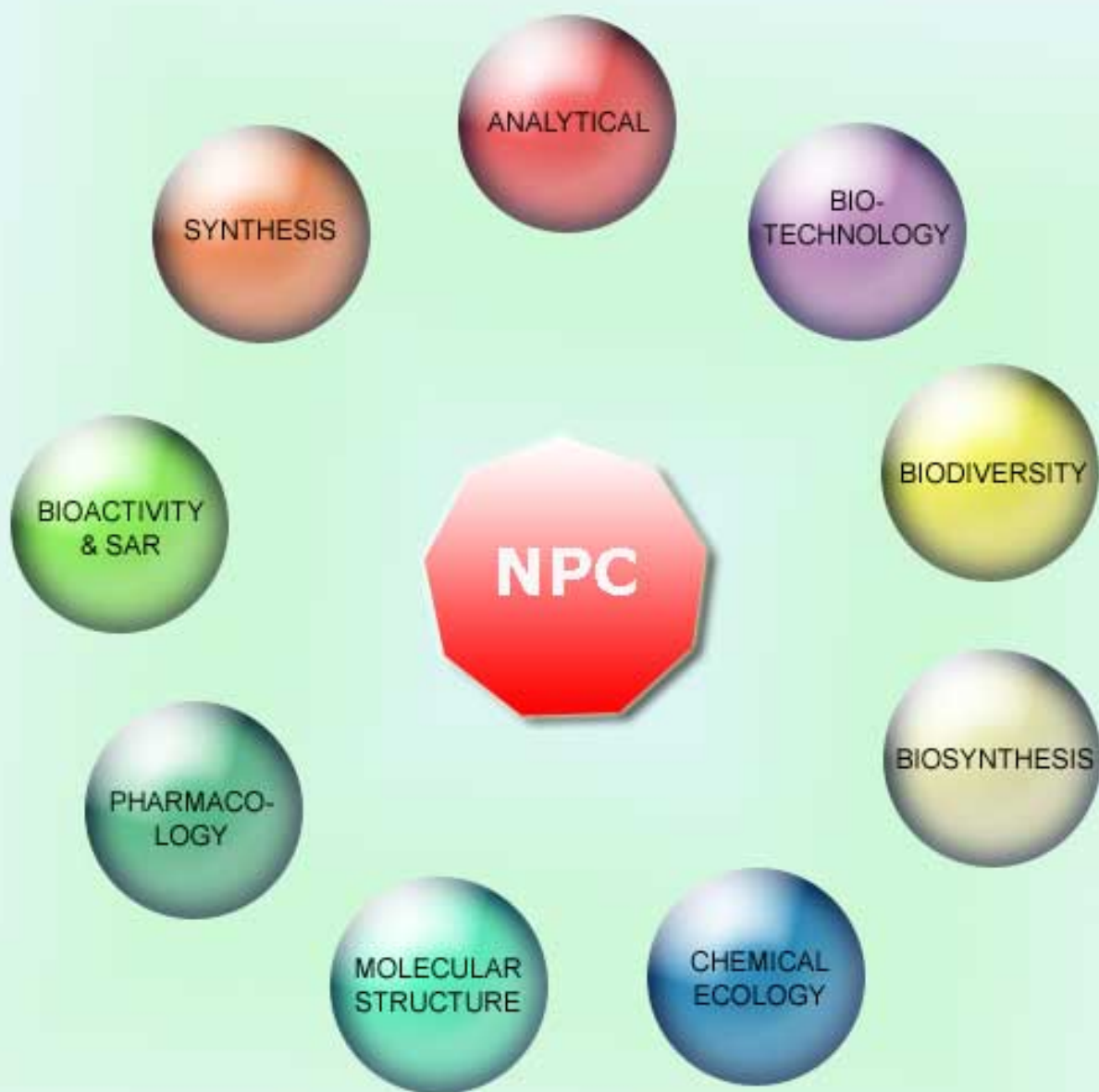
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on the Occasion of his 70th Birthday**

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Characterization of By-products of Saffron (*Crocus sativus* L.) Production

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The stigma, stamens and sepals of *Crocus sativus* L., from two different geographical origins, were analyzed for their crocin and flavonol contents. Identification of crocins, safranal, picrocrocin, and flavonols was carried out by HPLC/DAD and HPLC/MS analysis. Both stigma samples, grown under natural conditions, exhibited high crocin contents (between 342 and 231 mg/g), while the stamens and sepals were rich in flavonols (between 6 and 10 mg/g). The stamens contain mainly kaempferol- 3-*O*-sophoroside, whereas the sepals contain mainly quercetin and methyl-quercetin glycosides. These data may be useful in order to find a possible exploitation of the by-products of saffron production, in which large quantities of *C. sativus* flowers are available.

Keywords: Crocins, flavonols, HPLC/DAD/MS, sepals, stamens, stigma.

The dried, red stigmas of *Crocus sativus* L. are a very expensive spice known as saffron, which is used as a food flavoring and coloring agent and as a traditional herbal medicine [1a]. *Crocus* is cultivated in India, Iran, Spain, Greece and Italy. The production process involves a large amount of manual work and cannot be completely mechanized. In Italy, from a 1000 m² area, about 120,000-150,000 flowers can be obtained (4000-5000 kg), which give rise to 5-7 kg of fresh stigma, i.e. 1.0-1.3 kg of dried product.

Many papers deal with methods for the separation and determination of the biologically active [1b-1f] and aroma components [2a-2c]. The quality control of commercial saffron is checked using spectrophotometric [3a,3b], TLC [3c], GC [3d], HPLC [3e], and CE [3f] methods.

The purpose of this paper is the analysis of stigmas from *C. sativus* cultivated in Italy (Perugia and Fiesole) in order to characterize this commercial

saffron from a quality point of view. In these areas, cultivation is effected under natural conditions and without the use of any chemical product in the drying and conservation phases.

However, the most important part deals with the characterization of the biologically active components of the stamens and sepals in order to find a possible use for this material, which forms the major part of *C. sativus* flowers. The exploitation of stamens and sepals, notwithstanding their availability as by-products in the production of saffron, has not been taken into account, with the exception of one paper dealing with the isolation of flavonoids from crocus petals to study their tyrosinase inhibition action [4a]. Notwithstanding the lack of information on the polyphenol content of these tissues, petal extracts were used to control rat blood pressure [4b] and to test their antitussive effect in guinea pigs [1b]. The major biologically active components of saffron are crocin analogues, which are all glycosides of

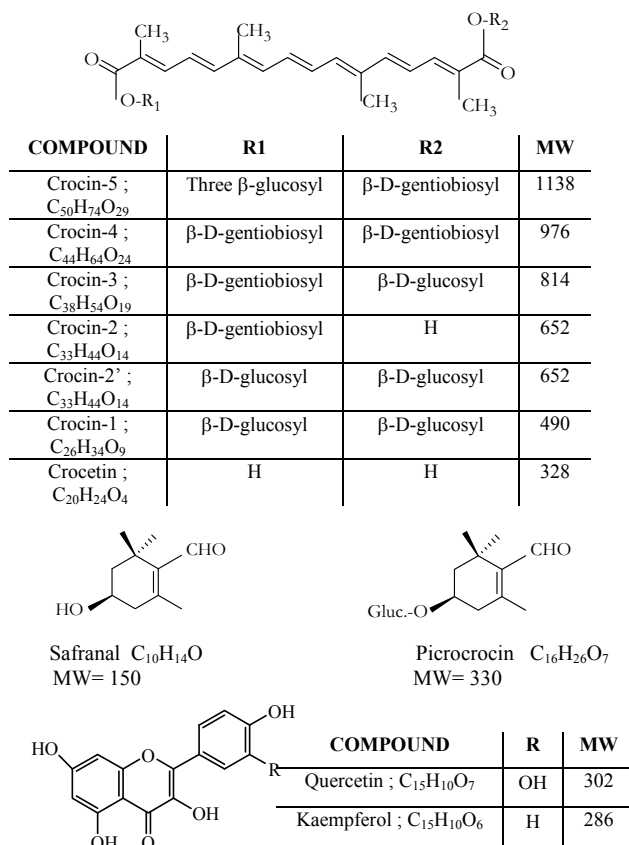


Figure 1: Chemical structures of saffron components

trans-crocetin, a carotenoid derivative, and which are responsible for the color. Safranal (2,6,6-trimethyl-1,3-cyclohexadien-1-carboxaldehyde), which is responsible for the characteristic aroma of saffron, is formed during storage by dehydration of picrocrocine, which is responsible for its bitter taste. Flavonoids are found in stigma, sepals, and stamens (Figure 1).

As regards stigma, the composition of the extract was similar to that found by other authors regarding crocins, picrocrocins, and safranal. Three kaempferol derivatives (two triglycosides and one diglycoside) were identified, according to previous findings [1f,5a]. In the case of stamens, a lesser number of crocins was found and quercetin, as well as kaempferol derivatives were detected. Also, methyl-quercetin derivatives in quite large amounts were recorded. There were no differences, from a qualitative point of view, between the two sampling zones; in fact only a quantitative variation was found in the samples from the different geographic regions [5a].

Table 1 reports the quantitative data for the dried stigma. It should be noted that the two samples differ are present in largest amount in the two samples. These compounds, together with *cis*-crocine 4, were

Table 1: Quantitative data for dried stigma. Average value ± SD of three samples. Data are expressed as mg/g fresh sample.

COMPOUNDS (Rt)	Stigma (FI)	Stigma (PG)
<i>trans</i> crocin-5 (10.30)	2.4±0.09	2.0±0.07
crocine derivative (11.14)	2.1±0.08	0.8±0.04
crocine derivative (11.46)	0.3±0.01	0.3±0.01
crocine derivative (11.87)	0.3±0.009	0.1±0.007
<i>trans</i> crocin-4 (12.84)	238.9±2.86	148.5±2.66
crocine derivative (13.88)	1.3±0.06	0.5±0.02
<i>trans</i> crocin-3 (14.39)	65.6±1.84	46.2±1.38
crocine derivative (14.99)	0.2±0.01	0.2±0.009
crocine derivative (15.90)	0.6±0.03	0.5±0.02
<i>trans</i> crocin-2' (16.17)	2.1±0.07	1.5±0.06
crocine derivative (17.37)	0.3±0.01	0.3±0.009
<i>cis</i> crocin-4 (17.79)	9.5±0.33	14.1±0.49
<i>trans</i> crocin-2 (19.33)	16.9±0.51	14.8±0.50
crocine derivative (20.40)	0.2±0.009	traces
crocine derivative (21.11)	0.3±0.01	traces
<i>cis</i> crocin-1 (22.02)	1.0±0.05	0.8±0.04
crocine derivative (22.81)	0.2±0.01	traces
crocine derivative (23.17)	traces	0.5±0.02
TOTAL	342.02	231.1
Picrocrocine (6.34)	111.1±2.33	68.9±1.79
Safranal (24.87)	2.2±0.09	2.6±0.09
K-3-sophorose -7- glucoside (3.78)	4.7±0.2	3.3±0.14
K -3,7,4'-triglucoside (5.90)	1.2±0.05	0.9±0.04
K-3-sophorose (8.49)	6.2±0.22	5.4±0.17
TOTAL	12.1	9.64

mainly in *trans*-crocine 4, *trans*-crocine 3 and picrocrocine contents, i.e. the three compounds which also the main compounds found by Caballero-Ortega *et al.* [5b] in a study of 11 saffron samples from different origins. The crocins content of the two samples is quite high giving evidence for the very good quality of the two samples. Among flavonols, kaempferol-3-*O*-sophorose was the main compound reported for a Spanish sample analyzed by Carmona *et al.* [5a].

Table 2 reports the crocin contents of sepals and stamens. The amount of crocins is low, while that of flavonols (Table 3) ranged from 10.1 to 6.1 mg/g. Stamens and sepals differ mainly in their kaempferol-3-*O*-sophorose content, which is the most abundant flavonol in the sepals.

The flavonols composition of the two tissues is different: in sepals, kaempferol derivatives ranged between 91 -93 %, whereas in stamens, quercetin and methyl-quercetin derivatives ranged between 52-71%. From all these data the possible exploitation of alternative tissues like stamens and sepals as phytochemical resources can be pointed out. For each kg of stigma, about 1000 kg of flowers are processed; therefore, sepals and stamens are important by-products of saffron production and their use could increase the economic value of *C. sativus* flowers.

Table 2: Crocins content of sepals and stamens. Average value \pm SD of three samples.

COMPOUNDS (Rt)	Sepals (FI)	Sepals (PG)	Stamens (FI)	Stamens (PG)
<i>trans</i> crocin-4 (12.84)	3.1 \pm 0.17	traces	112.2 \pm 5.65	4.0 \pm 0.19
crocins der. (13.88)			1.7 \pm 0.09	traces
<i>trans</i> crocin-3 (14.39)	0.8 \pm 0.04	traces	33.4 \pm 1.74	traces
crocins der. (14.99)			traces	traces
crocins der. (15.99)			1.3 \pm 0.07	
<i>trans</i> crocin-2' (16.17)			3.3 \pm 0.18	traces
<i>cis</i> crocin-4 (17.79)	traces		22.0 \pm 1.14	0.1 \pm 0.006
<i>trans</i> crocin-2 (19.33)	traces	traces	20.7 \pm 1.07	1.3 \pm 0.08
<i>cis</i> crocin-1 (22.02)			7.0 \pm 0.38	traces
crocins der. (22.81)			0.3 \pm 0.02	traces
crocins der. (23.17)			0.1 \pm 0.008	traces
<i>cis</i> crocin-2 (24.82)	0.3 \pm 0.02		0.6 \pm 0.03	
TOTAL	4.2	traces	196.3	5.4

Experimental

Sample preparation: Sepals, stamens and dried stigma samples were obtained from plants harvested in 2005 from Fiesole (FI, Italy) and Perugia (PG, Italy). Sepals and stamens (500 mg) were suspended in 50 mL of 70% ethanol, adjusted to pH 2.0 with formic acid, and left overnight. After extraction, the

samples were filtered to eliminate plant residues, and the filtrate evaporated to dryness under vacuum at room temperature. The residue was redissolved in EtOH/H₂O (70:30) and adjusted to pH 2.0 with formic acid to a final volume of 3 mL.

Saffron stigmas (50 mg) were extracted with 10 mL of 70% ethanol, adjusted to pH 2.0 with formic acid, left overnight and then filtered to eliminate plant residues. The extracts were analysed by HPLC/DAD/MS for the determination of saffron components.

Authentic standards of crocin were purchased from Fluka (St. Louis, USA), safranal from Sigma-Aldrich (St. Louis, USA), and *p*-hydroxybenzoic acid, kaempferol 3-*O*-glucoside, rutin and curcumin from Extrasynthèse S.A. (Lyon, France). All solvents were of HPLC grade purity (BDH Laboratory Supplies, United Kingdom).

HPLC/DAD analysis: Analysis for flavonols and crocins was carried out using a HP 1100L liquid chromatograph equipped with a DAD detector and managed by a HP 9000 workstation (Agilent Technologies, Palo Alto, CA, USA). Flavonols and crocins were separated by using a 150 \times 3.9 mm i.d. 4 μ m Nova-Pak C18 column (Waters) operating at 27°C. UV/Vis spectra were recorded in the 190-600 nm range and the chromatograms were acquired at 250, 308, 350 and 440 nm. The mobile phase was a

Table 3: Flavonols content of sepals and stamens. Average value \pm SD of three samples. Data are expressed as μ g/g fresh sample.

COMPOUNDS (Rt)	Sepals (FI)	Sepals (PG)	Stamen(FI)	Stamen(PG)
Kaempferol derivative (3.71)	76 \pm 4.10			
Kaempferol-3-sophoroside-7-glucoside (3.78)			511 \pm 22.84	923 \pm 41.53
Kaempferol derivative (5.81)			24 \pm 1.18	77 \pm 4.15
Kaempferol diglucoside (5.89)	97 \pm 4.85	113 \pm 5.6		
Kaempferol derivative (6.49)	15 \pm 1.03	34 \pm 1.83		
Kaempferol diglucoside (7.30)			416 \pm 21.16	755 \pm 33.75
Quercetin diglucoside (7.30)	480 \pm 22.08	738 \pm 32.16	1037 \pm 37.32	1227 \pm 47.81
Methyl quercetin diglucoside (7.82)	82 \pm 4.16	84 \pm 4.21	628 \pm 28.88	2091 \pm 61.74
Quercetin derivative (8.15)			27 \pm 1.15	39 \pm 2.14
Methyl quercetin di glucoside (8.42)			209 \pm 10.03	249 \pm 11.73
Kaempferol-3-sophoroside (8.49)	6415 \pm 192.45	8304 \pm 215.9	1702 \pm 64.7	377 \pm 17.72
Kaempferol glucosyl rhamnoside (9.29)	41 \pm 2.13	66 \pm 3.20		
Methyl quercetin derivative (9.34)			691 \pm 31.09	1188 \pm 46.32
Quercetin derivative (9.44)			239 \pm 11.47	303 \pm 14.54
Quercetin diglucoside (9.58)	24 \pm 1.27	60 \pm 3.18		
Kaempferol sinapoyl glucoside (10.59)	306 \pm 14.38	309 \pm 14.25	140 \pm 5.81	
Kaempferol derivative (10.86)				39 \pm 2.25
Kaempferol glucoside (10.98)	421 \pm 19.78	399 \pm 18.75	93 \pm 4.65	
Methyl quercetin glucoside (11.13)			52 \pm 2.75	176 \pm 8.62
Quercetin derivatives (11.55-12.21)			26 \pm 1.19	66 \pm 3.43
Kaempferol derivative (12.99)	21 \pm 1.15	17 \pm 0.078		
Quercetin <i>p</i> -cumaroyl glucoside (13.76)			199 \pm 9.75	237 \pm 110.61
Quercetin derivative (14.09)			4 \pm 0.22	26 \pm 1.20
Kaempferol <i>p</i> -cumaroyl glucoside (15.42)			35 \pm 2.05	52 \pm 2.65
Methyl quercetin <i>p</i> -cumaroyl glucoside (15.61)			26 \pm 1.21	40 \pm 2.12
Kaempferol (18.43)	20 \pm 1.16	14 \pm 0.74		8 \pm 0.44
TOTAL	7998	10138	6059	7873

one-step linear solvent gradient system, starting from 90% H₂O (adjusted to pH 3.2 with HCOOH) up to 100% CH₃CN during a 60-min period; flow rate 0.8 mL min⁻¹.

HPLC/MS analysis: HPLC/MS analysis was performed using a HP 1100L liquid chromatograph linked to a HP 1100 MSD mass spectrometer with an API/electrospray interface (Agilent Technologies, Palo Alto, CA, USA). The mass spectrometer operating conditions were: gas temperature, 350°C; nitrogen flow rate, 10.5 L/min, nebulizer pressure, 40 psi; quadrupole temperature, 30°C; and capillary voltage, 3500 V. The mass spectrometer was operated in positive mode at 120 eV.

Identification and quantification of individual polyphenols: Quantification of individual compounds was directly performed by HPLC/DAD using a five-point regression curve ($r^2 \geq 0.998$) in the range

0-30 µg on the basis of authentic standards. In particular, crocin derivatives were determined at 440 nm using curcumin as reference compound; safranal was determined at 308 nm using safranal as reference compound and picrocrocin was determined at 250 nm using *p*-hydroxybenzoic acid as reference compound. Flavonols, like kaempferol and quercetin derivatives, were determined at 350 nm using kaempferol-3-*O*-glucoside and rutin, respectively, as reference compounds. In all cases, actual concentrations of the derivatives were calculated after applying corrections for differences in molecular weight.

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Impurities in Herbal Substances, Herbal Preparations and Herbal Medicinal Products, IV.

Heavy (toxic) Metals

SFSTP Commission, Didier Guédon, Michèle Brum, Jean-Marc Seigneuret, Danièle Bizet, Serge Bizot, Edmond Bourny, Pierre-Albert Compagnon, Hélène Kergosien, Luis Georges Quintelas, Jérôme Respaud, Olivier Saperas, Khalil Taoubi and Pascale Urizzi

2107

A Fresh Insight into the Interaction of Natural Products with Pregnane X Receptor

Salvador Máñez

2123

Natural Products as Gastroprotective and Antiulcer Agents: Recent Developments

Rosa Tundis, Monica R Loizzo, Marco Bonesi, Federica Menichini, Filomena Conforti, Giancarlo Statti and Francesco Menichini

2129

Phytochemistry and Pharmacology of *Boronia pinnata* Sm.

Massimo Curini, Salvatore Genovese, Luigi Menghini, Maria Carla Marcotullio and Francesco Epifano

2145

Therapeutic Potential of *Kalanchoe* Species: Flavonoids and other Secondary Metabolites

Sônia S. Costa, Michelle F. Muzitano, Luiza M. M. Camargo and Marcela A. S. Coutinho

2151

<i>In vitro</i> Apoptotic Bioactivity of Flavonoids from <i>Astragalus verrucosus</i> Moris Joseph A. Buhagiar, Alessandra Bertoli, Marie Therese Camilleri-Podesta and Luisa Pistelli	2007
Qualitative Profile and Quantitative Determination of Flavonoids from <i>Crocus sativus</i> L. Petals by LC-MS/MS Paola Montoro, Carlo I. G. Tuberioso, Mariateresa Maldini, Paolo Cabras and Cosimo Pizza	2013
HPLC/DAD/ESI-MS Analysis of Non-volatile Constituents of Three Brazilian Chemotypes of <i>Lippia alba</i> (Mill.) N. E. Brown Patrícia Timóteo, Anastasia Karioti, Suzana G. Leitão, Franco Francesco Vincieri and Anna Rita Bilia	2017
Optimization and Validation of an HPLC–Method for Quality Control of <i>Pueraria lobata</i> Root Lidiya Bebrevska, Mart Theunis, Arnold Vlietinck, Luc Pieters and Sandra Apers	2021
Pharmacokinetics of Luteolin and Metabolites in Rats Sasiporn Sarawek, Hartmut Derendorf and Veronika Butterweck	2029
Complete Characterization of Extracts of <i>Onopordum illyricum</i> L. (Asteraceae) by HPLC/PDA/ESIMS and NMR Luisella Verotta, Laura Belvisi, Vittorio Bertacche and Maria Cecilia Loi	2037
Phenolic Profiles of Four Processed Tropical Green Leafy Vegetables Commonly Used as Food Sule Ola Salawu, Marzia Innocenti, Catia Giaccherini, Afolabi Akintunde Akindahunsi and Nadia Mulinacci	2043
(Bio)Sensor Approach in the Evaluation of Polyphenols in Vegetal Matrices M. Camilla Bergonzi, Maria Minunni and Anna Rita Bilia	2049
<i>In vitro</i> Radical Scavenging and Anti-Yeast Activity of Extracts from Leaves of <i>Aloe</i> Species Growing in Congo Annalisa Romani, Pamela Vignolini, Laura Isolani, Sara Tombelli, Daniela Heimler, Benedetta Turchetti and Pietro Buzzini	2061
Antioxidant Principles and Volatile Constituents from the North-western Iberian mint “erva-peixeira”, <i>Mentha cervina</i> Matteo Politi, César L Rodrigues, Maria S Gão, Manuela E Pintado and Paula ML Castro	2065
Chemical Composition of <i>Thymus serrulatus</i> Hochst. ex Benth. Essential Oils from Ethiopia: a Statistical Approach Bruno Tirillini, Roberto Maria Pellegrino, Mario Chessa and Giorgio Pintore	2069
GC MS Analysis of the Volatile Constituents of Essential Oil and Aromatic Waters of <i>Artemisia annua</i> L. at Different Developmental Stages Anna Rita Bilia, Guido Flamini, Fabrizio Morgenni, Benedetta Isacchi and Franco Francesco Vincieri	2075
Do Non-Aromatic Labiatae Produce Essential Oil? The Case Study of <i>Prasium majus</i> L. Claudia Giuliani, Roberto Maria Pellegrino, Bruno Tirillini and Laura Maleci Bini	2079
Olive-oil Phenolics and Health: Potential Biological Properties Francesco Visioli, Francesca Ieri, Nadia Mulinacci, Franco F. Vincieri and Annalisa Romani	2085
Traceability of Secondary Metabolites in <i>Eucalyptus</i> and <i>Fagus</i> Wood derived Pulp and Fiber Aline Lamien-Meda, Karin Zitterl-Eglseer, Heidrun Fuchs and Chlodwig Franz	2089
Potential Anticancer Activity Against Human Epithelial Cancer Cells of <i>Peumus boldus</i> Leaf Extract Juan Garbarino, Nicolas Troncoso, Giuseppina Frasca, Venera Cardile and Alessandra Russo	2095
Antihyperalgesic Effect of <i>Eschscholzia californica</i> in Rat Models of Neuropathic Pain Elisa Vivoli, Anna Maidecchi, Anna Rita Bilia, Nicoletta Galeotti, Monica Norcini and Carla Ghelardini	2099
Problems in Evaluating Herbal Medicinal Products Jozef Corthout	2103

Natural Product Communications

2008

Volume 3, Number 12

Contents

	<i>Page</i>
1968-2008: 40 Years of Franco F. Vincieri's Natural Products Research Anna Rita Bilia	1941
Effects of Terpenoids from <i>Salvia willeana</i> in Delayed-type Hypersensitivity, Human Lymphocyte Proliferation and Cytokine Production Anna Vonaparti, Anastasia Karioti, Maria C. Recio, Salvador Máñez, José L. Ríos, Eleani Skaltsa and Rosa M. Giner	1953
Characterization of By-products of Saffron (<i>Crocus sativus</i> L.) Production Pamela Vignolini, Daniela Heimler, Patrizia Pinelli, Francesca Ieri, Arturo Sciullo and Annalisa Romani	1959
Antitrypanosomal and Antileishmanial Activities of Organic and Aqueous Extracts of <i>Artemisia annua</i> Anna Rita Bilia, Marcel Kaiser, Franco Francesco Vincieri and Deniz Tasdemir	1963
Secondary Metabolites from the Roots of <i>Salvia palaestina</i> Benth Antonio Vassallo, Ammar Bader, Alessandra Braca, Angela Bisio, Luca Rastrelli, Francesco De Simone and Nunziatina De Tommasi	1967
Cancer Chemopreventive Potential of Humulones and Isohumulones (Hops α- and Iso-α-acids): Induction of NAD(P)H:Quinone Reductase as a Novel Mechanism Gregor Bohr, Karin Klimo, Josef Zapp, Hans Becker and Clarissa Gerhäuser	1971
A Polar Cannabinoid from <i>Cannabis sativa</i> var. <i>Carma</i> Giovanni Appendino, Anna Giana, Simon Gibbons, Massimo Maffei, Giorgio Gnavi, Gianpaolo Grassi and Olov Sterner	1977
HPLC-DAD-MS Fingerprint of <i>Andrographis paniculata</i> (Burn. f.) Nees (Acanthaceae) Sabrina Arpini, Nicola Fuzzati, Andrea Giori, Emanuela Martino, Giacomo Mombelli, Luca Pagni and Giuseppe Ramaschi	1981
Diterpenoid Alkaloids and Phenol Glycosides from <i>Aconitum naviculare</i> (Brühl) Stapf. Stefano Dall'Acqua, Bharat B. Shrestha, Mohan Bikram Gewali, Pramod Kumar Jha, Maria Carrara and Gabbriella Innocenti	1985
Inhibition of PGHS-1 and PGHS-2 by Triterpenoid Acids from <i>Chaenomelis fructus</i> Eveline Reininger and Rudolf Bauer	1991
Preparative Isolation of Antimycobacterial Shoreic Acid from <i>Cabralea canjerana</i> by High Speed Countercurrent Chromatography Gilda G. Leitão, Lisandra F. Abreu, Fernanda N. Costa, Thiago B. Brum, Daniela Fernandes Ramos, Pedro Eduardo A. Silva, Maria Cristina S. Lourenço and Suzana G. Leitão	1995
Antiplasmodial Effects of a few Selected Natural Flavonoids and their Modulation of Artemisinin Activity Anna Rita Bilia, Anna Rosa Sannella, Franco Francesco Vincieri, Luigi Messori, Angela Casini, Chiara Gabbiani, Carlo Severini and Giancarlo Majori	1999
Comparative Analysis of Antimalarial Principles in <i>Artemisia annua</i> L. Herbal Drugs from East Africa Silvia Lapenna, Maria Camilla Bergonzi, Franco Francesco Vincieri and Anna Rita Bilia	2003

(Continued inside backcover)